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Callus induction, plant regeneration and somaclonal variation in *in vivo* and *in vitro* grown White shrimp plant (*Justicia betonica* Linn.).

Jamilah Syafawati Yaacob*, Rosna Mat Taha, Norizam Jaafar, Zuliana Hasni, Hashimah Elias, Normadiha Mohamed

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

***Corresponding author: jam_sya@yahoo.com**

Abstract

Justicia betonica Linn. is traditionally used to treat various illness like cough, scaly skin and as an erosion control plant. This paper reports on callus induction and *in vitro* propagation of *J. betonica* from 3-week-old petiole and internode explants of this species. Callus was readily induced from petiole explants when cultured on Murashige and Skoog's medium (MS) fortified with NAA (0.5 - 2.0 mg L^{-1}), BAP (0.5 - 2.0 mg L^{-1}), Kinetin (1.0 - 2.0 mg L^{-1}) and Zeatin (1.0 - 2.0 mg L^{-1}), while internode explants only showed formation of callus on MS basal and when 0.5 mg L^{-1} BAP or 1.5 mg L^{-1} Kinetin were added. Optimum *in vitro* regeneration in *J. betonica* had been successfully achieved using internode explants cultured on MS medium supplemented with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. Rhizogenesis was observed from petiole cultures supplemented with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP as well as on MS with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP, where the latter had shown the most optimum response, with percentage of root formation of 66.67 ± 5.36%. More hormone treatments were observed to yield rhizogenesis from internode explants, especially when cultures were fortified with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP. Genetic stability in *in vitro J. betonica* plants was observed by studying the variation in mitotic index and chromosome numbers using cytological data. Similar values of mean Mitotic index (MI) and mean chromosome numbers ($2n = 14$) indicated that the transfer from *in vivo* to *in vitro* environment had no significant effect on genetic stability of this plant. However, the mean nuclear size was found to increase, while the mean cell size was reduced in *in vitro J. betonica* compared to *in vivo* plants. *In vitro* regenerated *J. betonica* plants were clonally uniform and showed no distinct morphological abnormalities, indicating the lack of somaclonal variation.

Keywords: callus induction; *Justicia betonica* Linn.; cellular behaviour; mitotic index; somaclonal variation.
Abbreviations: BAP - 6-benzyl aminopurine; NAA - α-naphthalene acetic acid; Kinetin - 6-furfurylaminopurine.

Introduction

Justicia betonica Linn. is a dicotyledonous plant and a member of the family Acanthaceae. It is well known in Malaysia as 'Ekor Tupai' (Burkill, 1985) or 'White Shrimp Plant' in English (Hsuan, 1986). *J. betonica* is a glabrous shrub commonly found in rocky places and waste lands, with white-green flowers and ability to withstand extreme environmental conditions such as drought and overflow of water or flood. *J. betonica* was also reported to contain several compounds with medicinal values, for instance the Sukuma people in Tanganyika used the mixture of butter and *J. betonica* plant ash as ointment to treat scaly skin (Burkill, 1985), while in Kenya and India, the inflorescence, flower ash and leaves mixture were used to treat coughing (Pascaline et al., 2008) and for hair wash (Rao et al., 2006).
Recently, *J. betonica* had been widely introduced as vegetation to promote slope stability and as an erosion control plant, especially in parts of Malaysia (Osman and Barakbah, 2011). This is due to the extensive root system of *J. betonica* that are fast-growing, with good plant-water relations and the fact that this species is self-sustainable. The seeds of *J. betonica* are quite difficult to be obtained, thus conventional propagation of this species had been carried out via stem cuttings. However, the prospect of utilizing *J. betonica* for erosion control requires rapid propagation of this species, which is made possible via micropropagation through tissue culture.

The present study not only reports on tissue culture and induction of callus in *J. betonica*, but also on cellular behaviour studies such as Mitotic index (MI) detection, mean cell and nuclear areas and chromosome counts from plants grown *in vivo* and *in vitro*. Cytological studies can help to detect somaclonal variations and deduce the genetic stability of a plant when subjected to tissue culture system (Raha and Roy, 2003). Measurement of cellular parameters is also important in distinguishing embryogenic from non-embryogenic callus (Moghaddam and Taha, 2005), as well as providing valuable insights in chromosomal studies, correlation of cell division with growth rates of plants (Ekanem and Osuji, 2006) and also during *in vitro* flowering (Taha and Wafa, 2012).

Results

Induction of callus from petiole explants

In general, *in vitro* culture of petiole explants on MS medium supplemented with NAA and BAP resulted in formation of callus after 5 weeks of culture (Table 1), in contrast to *in vitro* culture of internode explants, which showed callus formation after 1 week (Table 2). It was found that combinations of 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP were the most suitable for production of callus from petiole

explants of *J. betonica*, with 30.48 ± 0.55% of the explants produced callus, while combinations of high concentrations of NAA (1.5 – 2.0 mg L^{-1}) and BAP yielded no response (Table 1). Additions of low concentrations of NAA (0.5 mg L^{-1}) with BAP also yielded no response, except on MS medium fortified with 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} BAP (Table 1). *In vitro* cultures of *J. betonica* petiole explants produced white callus, except on petioles cultured on MS supplemented with 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} BAP which produced green callus. On the other hand, formation of callus from internode explants was only achived on MS basal medium and when 0.5 mg L^{-1} BAP or 1.5 mg L^{-1} Kinetin was added (Table 2). Rhizogenesis or root organogenesis was observed from callus derived from petiole explants cultured on MS supplemented with combinations of 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP as well as on MS added with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP (Table 1). Furthermore, combinations of 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP were also found to be the best for production of roots from petiole explants of *J. betonica*, whereby 66.67 ± 5.36% of the explants produced roots (Table 1). More hormone treatments were observed to yield rhizogenesis from internode explants, especially when cultures were fortified with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP (Table 2). All hormone treatments tested in the present investigation showed no formation of shoots when petiole explants were used, however plantlet regeneration in *J. betonica* had been successfully achieved using internode explants cultured on MS medium fortified with 0.5 – 1.5 mg L^{-1} NAA and 0.5 – 2.0 mg L^{-1} BAP (Table 2). MS medium supplemented with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP was the most effective in plantlet regeneration from internode explants of *J. betonica* (Fig 1), with 41.74 ± 0.17% explants produced shoots and 12.33 ± 0.35% explants produced roots. It was found that only 29.03% of all hormone treatments had resulted in formations of callus from petiole explants, while the majority of hormone treatments yielded no response. Root organogenesis was also only observed in petiole cultures grown on two media compositions (Table 1). The lack of response to hormone treatments in *J. betonica* Linn. and its difficulty to regenerate *in vitro* was found to correlate with its low Mitotic index (MI) and other factors. Organogenesis such as formation of roots in *J. betonica* was also a factor in lowering the MI values of meristematic cells of this species. Formation of callus and root organogenesis from petiole explants of *J. betonica* are shown in Fig 1.

Morphology of in vivo and in vitro grown plants

Internode explants cultured on MS medium fortified with 0.5 - 1.5 mg L^{-1} NAA and 0.5 - 2.0 mg L^{-1} BAP showed formation of shoots and roots (Table 2), and the transfer to MS basal medium productively encouraged complete plant regeneration of *J. betonica*. The regenerants were acclimatized *ex vitro* on black (peat) soil, followed by transfer to the greenhouse and eventually to the natural environment. It was observed that the leaves of *in vitro* grown plantlets started to fall off after *ex vitro* acclimatization, although soon replaced with new shoots that started to grow from the petioles after two weeks. The number of leaves was also found to increase after 5 weeks of transfer. The *ex vitro* plants also showed slower growth following acclimatization, only gaining about 5.4 cm in height after 5 weeks of transfer. Observations on the morphological characteristics of *in vivo* and *ex vitro* plants revealed no distinct variations. Both *in vivo* and *ex vitro J. betonica* leaves looked similar, with elliptical-shaped leaves that were opposite and green in colour. Moreover, the flowers of both *in vivo* and *ex vitro J. betonica* also showed similar morphology, where they bloomed on upright spires and consist of papery, green-veined white bracts (Fig 2). The roots of *ex vitro J. betonica* also appeared morphologically similar to roots of *in vivo* plants, whereby the roots were long, thin and hairy (Fig 3). However, the *ex vitro* roots appeared denser than roots of *in vivo* plants, possibly due to the additions of plant hormones during tissue culture protocols (Fig 3).

Cytological studies of in vivo and in vitro grown plants

Permanent slides of 1-week-old primary roots of *in vivo* and 3-month-old *in vitro* grown *J. betonica* were prepared as previously described. Cytological experiments conducted on those samples revealed slightly different mean MI values for both *in vivo* and *in vitro* grown *J. betonica* meristematic cells, with mean MI values of 27.76 ± 1.43% and 25.34 ± 2.4%, respectively (Table 3). The mean number of chromosomes for both *in vivo* and *in vitro* grown *J. betonica* was also similar, with 14 chromosomes per meristematic cell (Table 3). Feulgen-stained meristematic cells of *in vivo* and *in vitro J. betonica*, showing 14 chromosomes per cell are shown in Fig 4. However, the size of meristematic cells of *in vitro* grown *J. betonica* was small and had bigger nucleus than *in vivo* plants (Table 3), with mean cell area and nuclear area of 167.46 ± 49.39 μm^2 and 20.16 ± 0.89 μm^2, respectively. In contrast, the cell size of *in vivo J. betonica* was 347.56 ± 163.59 μm^2, with small nucleus (15.97 ± 0.69 μm^2). The ratio of mean nuclear area to mean cell area (N/C) was also different (Table 3), with N/C ratio of 0.12 ± 0.02 for *in vitro* grown plants, double than that recorded for *in vivo* plants (0.05 ± 0.01).

Discussion

We found that MS medium supplemented with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP yielded excellent results in tissue culture of *J. betonica*, showing optimum rhizogenesis and callus formation from petiole explants and optimum plantlet regeneration from internode explants. The effectiveness of NAA and BAP in induction of callus from petiole explants of *J. betonica* was corroborated by the findings of Ahmad and Spoor (1999) in their work on Curly kale (*Brassica oleraces* L.), where they reported that NAA and BAP combined had enhanced the production of callus in *Brassica oleraces*, especially when used in NAA : BAP at 1:1 ratio. It was found that high concentration of NAA (1.5 mg L^{-1}) with low concentration of BAP (0.5 mg L^{-1}) yielded the highest amount of callus in *in vitro* culture of *J. betonica* petiole explants, while resulted in formation of shoots and roots from cultures of internode explants. Contrasting results were reported on tissue culture of *Solanum melongena* L., where callus formation was most optimum when MS medium was supplemented with low concentration of NAA and high concentration of BAP (Ray et al., 2011). On the other hand, Devendra et al. (2011) also reported an increase in the frequency of callus formation from nodal explants of *Eclipta alba* L. Hassk when concentrations of NAA were increased. Besides, NAA and BAP were shown to promote shoot growth and proliferation of pineapple (Al-Saif et al., 2011) in addition to formation of multiple shoots and subsequent plantlet regeneration from stems of *Lawsonia inermis* syn. *L. alba* or henna plant (Rahiman and Taha 2011). Cytological studies are mainly conducted to evaluate and determine DNA content, chromosome count, genetic stability and cell cycle (Gould, 1984). The transfer of cells from one environment to

Table 1. Callus induction and rhizogenesis from petiole explants of *Justicia betonica* Linn. cultured on MS medium supplemented with various hormones after 3 months of culture.

[NAA] mg L^{-1}	[BAP] mg L^{-1}	[Kinetin] mg L^{-1}	[Zeatin] mg L^{-1}	Root formation (%)	Callus formation (%)	Colour of callus	Observations
0.0	0.0			NR	1.46 ± 0.11^{a}	White	Formation of callus after 60 days
	0.5			NR	1.12 ± 0.24^{a}	White	Formation of callus after 50 days
	1.0			NR	NR	N/A	No reponse, eventually explant became necrotic
	1.5			NR	16.17 ± 2.11^{d}	White	Formation of callus after 50 days
	2.0			NR	NR	N/A	No reponse, eventually explant became necrotic
0.5	0.0			NR	NR	N/A	No reponse, eventually explant became necrotic
	0.5			NR	NR	N/A	No reponse, eventually explant became necrotic
	1.0			NR	NR	N/A	No reponse, eventually explant became necrotic
	1.5			NR	NR	N/A	No reponse, eventually explant became necrotic
	2.0			NR	11.02 ± 0.02^{c}	Green	Formation of callus after 44 days
1.0	0.0			NR	9.07 ± 0.11^{c}	White	Formation of callus after 38 days
	0.5			NR	NR	N/A	No reponse, eventually explant became necrotic
	1.0			6.59 ± 0.05^{a}	4.43 ± 0.20^{b}	White	Formation of callus after 50 days
	1.5			NR	NR	N/A	No reponse, eventually explant became necrotic
	2.0			NR	NR	N/A	No reponse, eventually explant became necrotic
1.5	0.0			NR	NR	N/A	No reponse, eventually explant became necrotic
	0.5			66.67 ± 5.36^{b}	30.48 ± 0.55^{e}	White	Formation of callus after 48 days
	1.0			NR	NR	N/A	No reponse, eventually explant became necrotic
	1.5			NR	NR	N/A	No reponse, eventually explant became necrotic
	2.0			NR	NR	N/A	No reponse, eventually explant became necrotic
2.0	0.0			NR	NR	N/A	No reponse, eventually explant became necrotic
	0.5			NR	NR	N/A	No reponse, eventually explant became necrotic
	1.0			NR	NR	N/A	No reponse, eventually explant became necrotic
	1.5			NR	NR	N/A	No reponse, eventually explant became necrotic
	2.0			NR	NR	N/A	No reponse, eventually explant became necrotic
		1.0	0.0	NR	NR	N/A	No reponse, eventually explant became necrotic
		1.5		NR	4.78 ± 0.46^{b}	White	Formation of callus after 50 days
		2.0		NR	NR	N/A	No reponse, eventually explant became necrotic
		0.0	1.0	NR	NR	N/A	No reponse, eventually explant became necrotic
			1.5	NR	0.49 ± 0.15^{a}	White	Formation of callus after 48 days
			2.0	NR	NR	N/A	No reponse, eventually explant became necrotic

*means with different letters in the same column differ significantly at $p < 0.05$, by one way ANOVA and Duncan's multiple range test. [%: Percentage of (no of explants producing callus and roots / *n*), NR: No response, N/A: Not available]

a different environment can cause changes in cell activities (Thomas and Davidson, 1983), for example the transfer from *in vivo* to *in vitro* conditions can result in changes of cellular behaviour (Amstrong and Francis, 1987). In the present investigation, it was found that mean cell area of *in vitro* grown *J. betonica* was smaller than *in vivo* grown *J. betonica*, while mean nuclear area was found to be bigger in *in vitro* plantlets than *in vivo* plants indicating an unstable cellular activity due to tissue culture stress. Extensive studies had been conducted on nuclear and cell sizes of higher plants (Lyndon, 1967; Thomas and Davidson, 1983), although very few published work was found for tissue culture-derived plants. According to Bayliss (1985), plants originated from tissue culture usually have a longer cell cycle, which in turn may cause some changes in DNA content as well as cell and nuclear sizes, due to the transfer from *in vivo* to *in vitro* conditions. The reduced mean cell area and the increase in mean nuclear area showed by the meristematic cells of *in vitro* grown *J. betonica* indicated an unstable cellular activity, which might have been triggered due to tissue culture stress. It was possible that the mechanisms involved in nuclear and cell area determination was interrupted when the cells of

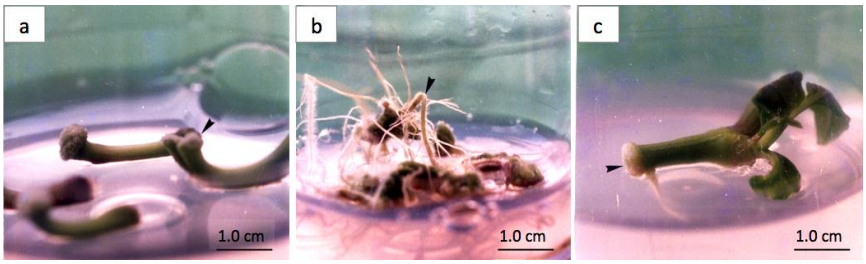

Fig 1. Effects of NAA (1.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) on different explants of *Justicia betonica*. **(a)** Petiole explants started to show formation of white callus after 48 days of culture and **(b)** showed optimum root organogenesis with the highest percentage of root formation. **(c)** Direct regeneration of shoots was also achieved when internode explants were cultured on similar growth medium, indicating the effectiveness of 1.5 mg L^{-1} NAA and 1.5 mg L^{-1} NAA combined in callus induction and plant regeneration in *J. betonica*. Arrows show the formation of callus and subsequent root organogenesis on petiole explants of *J. betonica*.

intact *J. betonica* entered tissue culture system. Thomas and Davidson (1983) and Taha and Francis (1991) also stated that the 'steady-state' condition of the cells might change as a result of the transfer from *in vivo* to *in vitro* environment. Taha and Wafa (2012) also reported lower mean cell areas but higher nuclear areas in *in vitro* grown *Celosia cristata*. However, contrasting results were reported for *Cocos nucifera* L., whereby callus-derived mitotic cells of this species were bigger than root-derived meristematic cells (Amstrong and Francis, 1987). Van Zeist et al. (1990) also reported similar results from their work on *Zea mays* L., suggesting the involvement of a specific mechanism that can trigger a change in cellular activity of cells cultured *in vitro*. However, Cavalier-Smith (1982) and Jordan et al. (1987) suggested a correlation between cell size and nuclear size, although both parameters are directly dependent on DNA content. Organogenesis such as formation of shoots or roots was reported to influence the reduction of cell size in tissue culture system. In the present study, cytological analysis was conducted on meristematic cells of roots derived from callus of *J. betonica*, suggesting that root organogenesis might have been a contributing factor to the decrease of cell size in *in vitro* grown *J. betonica* compared to *in vivo* plants. Similarly, Ross et al. (1973) reported a reduction in cell size of callus-derived meristematic cells of tobacco. Moreover, Thomas and Davidson (1983) also reported an interchanging relationship between nuclear and cell size of *Vicia faba* which can undergo abrupt changes without affecting mitotic activity, in agreement with the findings of current investigation which showed similar MI values between *in vivo* and *in vitro J. betonica*, despite the striking differences in nuclear and cell size. The mean MI values for both *in vivo* and *in vitro* grown *J. betonica* meristematic cells were slightly different, with mean MI values of 27.76 ± 1.43% and 25.34 ± 2.4%, respectively (Table 3). The mean MI value of *in vitro* meristematic cells was slightly lower than *in vivo*, however the differences were found to be statistically insignificant ($P > 0.05$). This indicates that the transfer into tissue culture system did not affect cell divisions in *J. betonica*, as it was found that mitosis occurred at a similar rate *in vitro* and *in vivo*. In contrast, Ramulu et al. (1985) and Taha and Francis (1990) reported lower MI values in *in vitro* grown *Solanum tuberosum* and *Vicia faba* than *in vivo* grown plants. The low MI values observed in *in vitro* grown *J. betonica* was similar to the MI value recorded for *Vicia faba* (25%), correlated with the difficulty in callus induction and *in vitro* regeneration of *Vicia faba* (Taha and Francis, 1990). This is consistent with the observations recorded for *J. betonica*, where it was found that *in vitro* culture of petiole explants of this species only produced callus after 5 weeks of culture and failed to yield any formation of direct shoots (Table 1). Root organogenesis was also observed only from petiole cultures supplemented with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP as well as on MS fortified with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. However, there are other factors that can influence the Mitotic index of a species, for example, Tabur and Oney (2009) stated that additions of high concencentrations of fertilizers had a negative influence on chromosome behaviour and Mitotic index of *Vicia hybrida* L. In addition, the mean number of chromosomes for both *in vivo* and *in vitro* grown *J. betonica* was also found to be similar, with 14 chromosomes per cell (Table 3). This is in agreement with the findings of Darlington and Wylie (1955), where they reported *Justicia* sp. had 14 chromosomes (diploid). In general, chromosomal abnormality could be caused by prolonged duration of tissue culture, subsequently causing some plants to lose their morphogenetic potential (Torey, 1967; Evans et al., 1984). However, the decrease of embryogenesis potential in mature tissues is not influenced by DNA fragmentation and cell cycle (Taylor and Vasil, 1987), although genetic instability was found to correlate with endogenous growth factor within the tissues. Low concentrations of NAA were also reported to influence cell shape and chromosomal numbers (Muir, 1980), for instance, Ogura (1982) argued that additions of auxin and cytokinins played an important role in the change of chromosomal numbers in *Vicia faba*. Nevertheless, there are other contributing factors involved in the occurrence of somaclonal variations in tissue culture-derived plants, such as the compositions of growth media, culture time (Varga et al., 1988) and even due to the variations in gene expression during *in vitro* culture and plant regeneration stage (Evans and Bravo, 1986; Karp, 1989). In the current work, analysis of results showed that no somaclonal variations had occurred in *in vitro* grown *J. betonica*. Visual observations conducted on both *ex vitro* and *in vivo J. betonica* revealed no distinct abnormalities, whereby both *in vivo* and *ex vitro* plants appeared morphologically similar (Fig 1). Cellular parameters measured in this study also supported this finding, as elucidated by similar mean MI values and chromosome numbers between *in vivo* and *in vitro* grown *J. betonica*. However, further researches are in progress to determine the effects of culture time and other growth hormones on genetic stability of *J. betonica* when cultured *in vitro*.

Materials and methods

Plant material and induction of callus

Stem cuttings from intact plants of *J. betonica* Linn. were grown on black (peat) soil and explants (petiole, internode) were excised from 3-week-old intact *J. betonica* plants to

Table 2. Callus induction and plant regeneration from internode explants of *Justicia betonica* Linn. cultured on MS medium supplemented with various hormones after 3 months of culture.

[NAA] mg L^{-1}	[BAP] mg L^{-1}	[Kinetin] mg L^{-1}	[Zeatin] mg L^{-1}	Root formation (%)	Shoot formation (%)	Callus formation (%)	Colour of callus	Observations
0.0	0.0			NR	NR	47.66 ± 0.46^{b}	White	Formation of callus after 10 days
	0.5			NR	NR	44.78 ± 0.65^{b}	White	Formation of callus after 8 days
	1.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
	1.5			34.65 ± 0.71^{c}	NR	NR	N/A	Formation of roots after 46 days
	2.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
0.5	0.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
	0.5			13.42 ± 0.14^{a}	25.64 ± 0.44^{c}	NR	N/A	Plantlet regeneration after 30 days
	1.0			47.44 ± 0.31^{d}	NR	NR	N/A	Formation of roots after 9 days
	1.5			34.51 ± 0.12^{c}	NR	NR	N/A	Formation of roots after 9 days
	2.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
1.0	0.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
	0.5			10.45 ± 0.11^{a}	26.15 ± 0.14^{c}	NR	N/A	Plantlet regeneration after 38 days
	1.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
	1.5			24.56 ± 0.26^{b}	12.54 ± 0.35^{b}	NR	N/A	Plantlet regeneration after 46 days
	2.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
1.5	0.0			10.43 ± 0.71^{a}	NR	NR	N/A	Formation of roots after 50 days
	0.5			12.33 ± 0.35^{a}	41.74 ± 0.17^{d}	NR	N/A	Plantlet regeneration after 35 days
	1.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
	1.5			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
	2.0			24.56 ± 0.26^{b}	21.46 ± 0.18^{c}	NR	N/A	Plantlet regeneration after 40 days
2.0	0.0			11.44 ± 1.14^{a}	NR	NR	N/A	Formation of roots after 40 days
	0.5			24.54 ± 0.19^{b}	NR	NR	N/A	Formation of roots after 40 days
	1.0			15.48 ± 1.12^{a}	NR	NR	N/A	Formation of roots after 60 days
	1.5			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
	2.0			NR	NR	NR	N/A	No reponse, eventually explant became necrotic
		1.0		NR	20.83 ± 2.44^{c}	NR	N/A	Formation of shoots after 30 days
		1.5	0.0	NR	25.84 ± 0.57^{c}	3.75 ± 0.55^{a}	White	Formation of callus and plantlet regeneration after 62 days
		2.0		NR	8.33 ± 1.41^{a}	NR	N/A	Formation of shoots after 64 days
		0.0	1.0	NR	NR	NR	N/A	No reponse, eventually explant became necrotic
			1.5	NR	4.71 ± 2.14^{a}	NR	N/A	Formation of shoots after 90 days
			2.0	NR	NR	NR	N/A	No reponse, eventually explant became necrotic

*means with different letters in the same column differ significantly at $p < 0.05$, by one way ANOVA and Duncan's multiple range test.
[%: Percentage of (no of explants producing callus and roots / *n*), NR: No response, N/A: Not available]

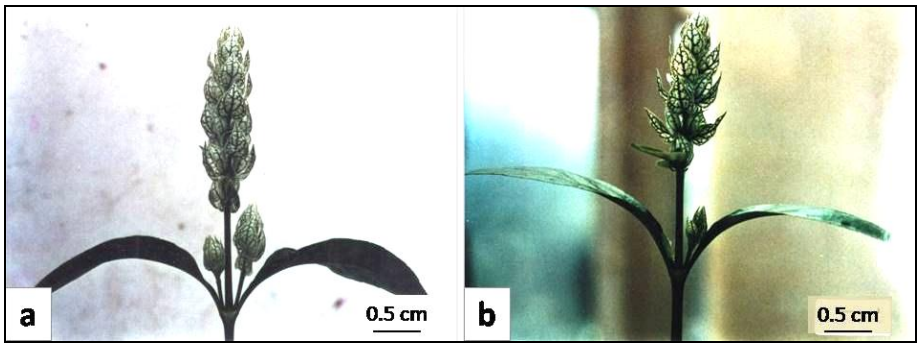

Fig 2. Flowers of *in vivo* **(a)** and *in vitro* **(b)** grown *Justicia betonica*, showing similar morphological characteristics. Flowers and leaves appeared identical, indicating the lack of somaclonal variation.

Table 3. Mean Mitotic index (MI), chromosome number, mean cell and nuclear areas of meristematic cells of *in vivo* and *in vitro* grown *Justicia betonica* Linn.

Cellular parameters	*Justicia betonica* Linn.	
	in vivo	*in vitro*
Mean Mitotic index (MI)	27.76 ± 14.3^{a}	25.34 ± 2.40^{a}
Chromosome number (mean)	$2n=2x=14^{a}$	$2n=2x=14^{a}$
Mean nuclear area (N)	15.97 ± 0.69^{a}	20.16 ± 0.89^{b}
Mean cell area (C)	347.56 ± 163.59^{b}	167.46 ± 49.39^{a}
Ratio (N/C)	0.05 ± 0.01^{a}	0.12 ± 0.02^{b}

*means with different letters in the same row differ significantly at $p < 0.05$, by one way ANOVA and Duncan's multiple range test.

initiate *in vitro* cultures of this species. Surface sterilizations of the explants were conducted following standard tissue culture protocols (Taha, 1993), but with several minor modifications. First, the explant segments were immersed in sterile distilled water added with 2-3 drops of Tween 20 to get rid of any adhering dirt and facilitate sterilization, followed by treatments with 70%, 50%, 30% and 10% (v/v) commercial bleach (Chlorox) for 3-4 minutes at each concentration. The explant segments were also rinsed 3 times using sterile distilled water after each treatment with commercial bleach. The sterilization procedure was completed by submerging the petiole segments in 70% (v/v) ethanol for a few seconds, followed by rinsing 3 times with sterile distilled water, conducted under aseptic conditions in a laminar flow chamber. The explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with various quality and quantity of plant growth regulators, such as NAA, BAP, Kinetin and Zeatin to induce production of callus and formation of roots of this species. Murashige and Skoog (MS) medium supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} agarose gel was used throughout the experiment, with pH of the medium adjusted to 5.8 ± 0.1 prior to autoclaving at 121 °C for 30 minutes. The cultures were maintained in the culture room at 25 ± 1°C with 16 hours light and 8 hours dark. The cultures were monitored and observations were recorded on weekly basis for 3 months.

Regeneration and morphology of in vitro and in vivo grown plants

Callus and explants showing formation of shoots and roots were transferred to plant growth regulator-free MS medium to encourage production of more shoots and roots and subsequently form complete plantlets. The MS basal medium was added with 30 g L^{-1} sucrose, pH 5.8 ± 0.1 and solidified with 8 g L^{-1} agarose gel. The cultures were maintained in the culture room at 25 ± 1°C with 16 hours light and 8 hours dark. Complete plantlets that formed after 3 weeks of culture were transferred to covered vases containing black (peat) soil and acclimatized in the culture room for one month. The plantlets were subsequently transferred to a green house, and their growth performance in the natural environment was monitored. The morphological features of both *in vivo* and *ex vitro J. betonica* were compared to explicate any morphological irregularities that might arise due to tissue culture stress. The shape of leaves, flowers, plant height and mean leaf diameter of the acclimatized *in vitro* grown *J. betonica* plantlets were measured and compared to *in vivo* plants of similar age (3-month-old). Root morphology (structure and thickness) of *in vivo* and *ex vitro* grown plants were also compared.

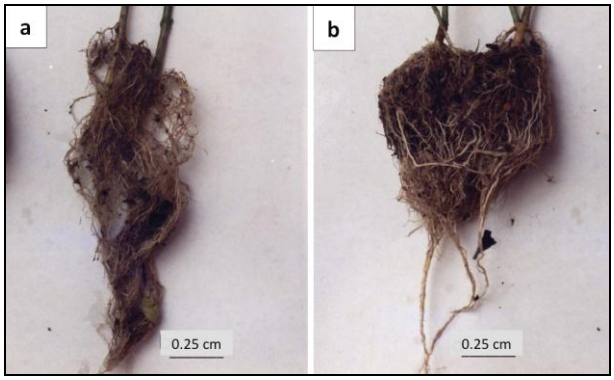

Fig 3. Root structures of in vivo (a) and in vitro (b) grown Justicia betonica, showing similar morphological characteristics. Roots appeared identical and fibrous, indicating the lack of somaclonal variation.

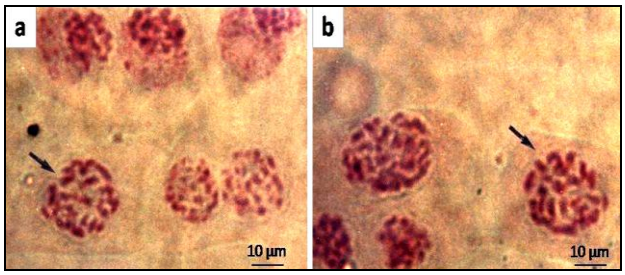

Fig 4. 14 chromosomes in a cell, as observed from squashed preparations of in vivo and in vitro Justicia betonica root tip meristems: (a) In vivo grown J. betonica. (b) In vitro grown J. betonica. Arrows show the stained chromosomes in J. betonica meristematic cell.

Cytological analysis on roots of in vivo and in vitro grown plants

One-week-old primary root segments of *in vivo* (standard growth) and roots from regenerated plants of *J. betonica* were stained using Feulgen and made into permanent slides, prior to cytological analysis to measure the Mitotic index (MI), chromosome number and mean nuclear and cell areas of this species. The root segments were excised and preserved overnight in 3:1 ratio of alcohol : acetic acid. Preparations of permanent slides were commenced by washing the root segments using distilled water for 5 minutes (twice), followed by immersion in 5M Hydrochloric acid (HCl) for 20 minutes.

The root segments were then stained with Feulgen (soaked for 2 hours) and stained root tips without the root caps were transferred onto glass slides, and added with 1-2 drops of 45% (v/v) acetic acid. The slides were then made permanent following the quick-freeze method as described by Conger and Fairchild (1953), and cover slides were mounted on the slides using DPX (Di-N-Butyle Phthalate in Xylene).

Cellular behaviour of *in vivo* and *in vitro* primary root cells of *J. betonica* was analyzed, whereby cellular parameters such as the Mitotic index (MI), chromosome count, mean nuclear and cell areas and their ratios were determined. Visualizations of the cells and chromosomes were conducted using a light microscope (Zeiss Axioscope, Germany) connected to a Sony video camera, supported by VIDAS (Kontron Electronic, Germany). Three permanent slides with at least 600 cells were observed to determine the Mitotic index, while at least 15 cells at metaphase spread were analyzed to determine the mean chromosome number of this species *in vivo* and *in vitro*.

Statistical analysis

Randomized complete block design (RCBD) with 30 replicates was employed in assessment and analysis of results, to decrease error and enhance accuracy. Statistical analysis was conducted using statistical variance test (ANOVA) and compared using Duncan's multiple range test (DMRT) with least significant differences at 5% level.

Conclusions

Formation of callus from petiole explants of 3-week-old *J. betonica* occurred after as early as 5 weeks, on MS medium supplemented with 1 mg L^{-1} NAA. In contrast, internode explants showed formation of callus after as early as 1 week when cultured on MS basal medium and when 0.5 mg L^{-1} BAP was added. However, only 29.03% of total hormone treatments had produced callus from petiole cultures, relatively better than internode cultures, which showed formation of callus on only three media compositions. Root organogenesis was observed from petiole-derived callus grown on MS supplemented with combinations of 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP and on MS with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP, with percentages of root formation of 6.59 ± 0.05% and 66.67 ± 5.36%, respectively. Root organogenesis was best achieved from petiole-derived callus grown on MS supplemented with 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP, the same media composition which showed optimum plantlet regeneration from internode explants. Cytological analysis conducted on root meristematic cells of *in vitro* and *in vivo* plants revealed similar values of mean Mitotic index (MI) and mean chromosome numbers, indicating that the transfer from *in vivo* to *in vitro* environment did not affect the genetic stability of this plant. However, the mean nuclear size increased, while the mean cell size was found to be reduced *in vitro*. Nevertheless, both *in vivo* and *in vitro J. betonica* showed similar morphological characteristics such as plant height, flower and leaf morphology and root structure, indicating no somaclonal variations had occurred during tissue culture and acclimatization process.

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